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ISFG: Recommendations regarding the use of non-human (animal) DNA in forensic genetic investigations

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ABSTRACT

The use of non-human DNA typing in forensic science investigations, and specifically that from animal DNA, is ever increasing. The term animal DNA in this document refers to animal species encountered in a forensic science examination but does not include human DNA. Non-human DNA may either be: the trade and possession of a species, or products derived from a species, which is contrary to legislation; as evidence where the crime is against a person or property; instances of animal cruelty; or where the animal is the offender. The first instance is addressed by determining the species present, and the other scenarios can often be addressed by assigning a DNA sample to a particular individual organism. Currently there is little standardization of methodologies used in the forensic analysis of animal DNA or in reporting styles. The recommendations in this document relate specifically to animal DNA that is integral to a forensic science investigation and are not relevant to the breeding of animals for commercial purposes. This DNA commission was formed out of discussions at the International Society for Forensic Genetics 23rd Congress in Buenos Aires to outline recommendations on the use of non-human DNA in a forensic science investigation. Due to the scope of non-human DNA typing that is possible, the remit of this commission is confined to animal DNA typing only.

1. Introduction

The aim of this commission is to make recommendations concerning standardization of DNA typing of animal species and products, other than human, in a forensic science investigation. Non-human DNA includes botanical and microbial taxonomic groups but, for the scope of this report, DNA typing will be focused on animal DNA; for simplicity animal DNA does not include that from humans.

There is an increasing interest in the forensic use of animal DNA. The issues addressed when using animal DNA are predominantly (i) the identification of the species and (ii) assigning samples to a particular individual.

1.1. Identification of the species

The loci most commonly used for species testing are the mitochondrial cytochrome *b* (cyt *b*) [1,2] and cytochrome oxidase gene loci (COI) [3]. The process of species identification is the same for either gene locus where a section of the gene is amplified and then directly sequenced. The cyt *b* gene has been a favored gene locus in taxonomy and species identification [1,2,4–8]. It has been applied to individual species identification such of many vertebrate species from sharks [9], snakes [10], marine turtles [11,12], to high profile species such as rhino [13], elephant ivory [14] and tigers [15,16]. Sequencing of a 600 base pair portion of the COI gene has been proposed as a means to catalogue the biodiversity on earth [3], and it has been used extensively in invertebrates [17,18] and fish [19–21]. In cases where differentiation of closely related species is required, D-loop sequences may be appropriate [1,22–25]. Guidelines for the use of mitochondrial DNA typing with regard to human identification using this

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mitochondrial locus have been developed and can be applied where appropriate [26–28].

The use of a single genetic marker may not yield high levels of confidence in taxonomic identification, and with the use of mitochondrial DNA it should be noted that hybrids would only share their type from the maternal donor. A sequence repository for both gene loci can be found on a DNA database such as GenBank (www.ncbi.nih.gov) or EMBL (www.embl.ac.uk).

1.2. Assignment of unidentified samples to a particular individual

The use of short tandem repeat (STR) loci is the more common method for the assigning of samples to an individual. STR allele frequency databases have been developed for a number of populations of mammalian species, particularly domesticated ones such as dogs [29–32], cats [33,34], horses [35–37], cows [38], and domestic pigeons [39]. For wild animals, there are few such databases in existence, but examples do exist including: deer species [40]; wild boars [41], tigers [42]; the European badger (*Meles meles*) [43]; birds of prey, such as the golden eagle (*Aquila chrysaetos*), and falcon species such as saker falcons (*Falco cherrug*) [44,45]; and rattle snakes (*Crotalus tigris*) [46]. It should be noted that these databases are for one population of a particular species and may, or may not, be relevant to other analyses of the same species.

Unlike the development of human DNA typing where the ISFG along with EDNAP, ENFSI, SWGDAM and other organizations has developed guidelines for nomenclature and relevant processes [47], the same is not the case in animal DNA typing for forensic purposes. Much is *ad hoc* in this field of research and professional work. There has been only one previous publication considering the standardization of methods in non-human DNA typing [48]. The present recommendation document is a starting point on the use of non-human DNA testing in a forensic investigation.

2. Sample collection

Investigations where the victim is an animal, or the trade in endangered species, may not have the same priority as crimes against people and property. However, the collection and packaging of the samples requires the same standards as the investigation of any incident. The integrity of the item and the traceability of evidence require the same attention to ensure that the developing case does not get challenged based on poor procedures.

Recommendation #1: *The same procedures to ensure integrity and traceability of the items should be employed in the collection and examination of animal samples as undertaken for any other forensic investigation.*

3. DNA samples used in validation studies

It is essential that the starting material from a reference sample is from a known source when performing validation studies. Voucher specimens can be obtained from zoological institutes and should be used where appropriate. If voucher specimens are not available, then authentication of the species through sequencing of a gene locus, such as the mitochondrial cyt *b* or COI genes, and comparison to sequences held on a repository such as GenBank, is acceptable. It should be noted in any subsequent report that GenBank is not regulated and that the sequence information obtained by such a comparison is assumed to be correct.

Recommendation #2: *Validation studies from non-domesticated species should use voucher specimens where possible. If this is not possible then a justification needs to be made for the sample type used.*

4. Species testing

A large number of primer sequences have been published as universal for different taxons. The genetic location of the primers should be recorded, specifying whether they are designed to be universal or species-specific. When universal primers are used, a potential admixture of DNA from further species must be taken into consideration – especially in cases when animal traces like small blood spots on human clothing are to be analyzed. If the universal primer preferentially amplifies human DNA under the given PCR conditions, the sequence of the questioned animal species will probably not be found. The position of the primers should be recorded in relation to the registered sequence on GenBank. In the case of loci on the mitochondrial genome, a complete genome sequence is preferable when designating the position of the primer. When a complete mitochondrial sequence is not known, the accession number of the sequence or the reference sequence should be provided. Confusion can occur if different nomenclature is used; hence agreement on nomenclature is needed at an early stage. The default mitochondrial DNA sequences for the canine mitochondrial D-loop have been developed [49] and serves as a model for future mitochondrial comparisons.

The specificity to the species under investigation needs to be shown for any primer set used, either by data provided or reference to a previously published article. Cross-reactivity studies should be performed and either available for external scrutiny or published in the public domain. Not only commonly occurring species should be chosen for cross-reactivity testing, but also any species that is considered genetically similar to the species in question.

The expected size of any amplicon should be recorded. This should be compared to the observed and any discrepancy should be accounted for.

The degree of homology with members of the same species should be recorded along with any intraspecies variation and the number of samples used in any intraspecies study. It may be that the DNA sequence lodged on GenBank is not representative of the species; this needs to be considered in any further study.

The difference between species with the closest genetic sequence should be recorded and any opportunity that intraspecies variation overlaps with interspecies variation should be noted. Such studies can lead to inaccurate conclusions that the results are in favor of the biological material coming from an individual of a certain species although the results might also be in favor of the material coming from an individual from another species.

Recommendation #3: *The choice of locus/loci used in species identification, such as, but not restricted to, the mitochondrial genes cyt b, COI, and the D-loop region, needs to be justified based on the ability to identify the unknown species among those that are close genetic relatives.*

Recommendation #4: *The nucleotide sequence and map showing the location of the primers used in species testing needs to be provided or referenced to a previously published article.*

Recommendation #5: *Intraspecies and interspecies studies should be provided for any novel primer set used in species identification. The process undertaken to validate the test should be provided, including, but not exclusively, studies on sensitivity, specificity, reproducibility and mixed samples.*

5. Primer design in identity testing

Primers designed to amplify repetitive DNA such as a short tandem repeat (STR) locus are normally taken from previous sequence data. The validity of the sequence data needs to be confirmed, particularly if taken from an open access database such as GenBank.

A demonstration of specificity to the species/genus/family to which the primers are designed is required. Cross-reactivity experiments should be performed and documented.

A number of samples, from the same sample set that are under investigation, need to be examined to ensure reproducibility and account for any variation within the sample set.

The process used to optimize a multiplex amplification needs to be provided.

The examination of a number of samples is also required to detect any ambiguities created by mutations in the primer sites. If possible, the priming sites should be sequenced and these sequences used should be made public. Priming sites for loci used in canine and feline testing have been lodged with STRBase (www.cstl.nist.gov/div831/strbase/).

Recommendation #6: *Primers used to amplify polymorphic DNA should be tested to ensure specificity and reproducibility and should be published in the public domain.*

6. Allele nomenclature designation for STR loci

The alleles obtained from any study of repetitive DNA should be sequenced; this is in line with a previous study of canine STR allele nomenclature [50] and follows on from guidelines developed by the ISFG [51]. For those involved with routine human identification in human criminal casework, there is familiarity with tetranucleotide repeats, although dinucleotide repeats have been used in previous non-human testing. Loci with two base repeats, however, suffer from increased stutter and altered heterozygote balance. Therefore, the use of dinucleotide repeats in forensic genetics is not recommended, except for those markers that are already used widely in animal genetic studies.

The percentage of stutter should be recorded; this is particularly important when dinucleotide repeat sequences are examined. Heterozygote balance should be reported for each locus tested. In the case of dinucleotide repeats, examples showing heterozygotes separated by one allele should be recorded to demonstrate how the alleles are designated.

Recommendation #7: *If repeat-based polymorphic loci are used for individualization, tetrameric short tandem repeat systems should be used preferentially.*

7. Allelic ladders for STR loci

The generation of an allelic ladder is the preferred means of designating alleles from questioned and known samples. A comprehensive survey of samples is required to generate a suitable allelic ladder such that the majority of questioned samples will fall within a known allele. The allelic ladder should preferentially contain sequenced alleles. A control DNA sample of known genotype needs to be used to confirm that the alleles separate as expected. If a species is to be the focus of on-going study then a default control DNA should be identified based on the method reported by Szibor et al. [52]. Depending upon the separation medium used, the allelic ladder will need to be analysed multiple times to detect electrophoretic variation. This will allow for $a \pm$ bp bin to be set at either side of the alleles within the allelic ladder for calling of unknown alleles either visually or by adopting relevant software. The use of an allelic ladder will permit the recording of STR alleles based on their repeat number rather than using size in base pairs.

Recommendation #8: *Sequenced allelic ladders are essential for the accurate designation of alleles and should be used in all STR typing. The number of repeats should be the basis of reporting of results rather than using only the size based on the number of base pairs of any samples tested.*

8. STR mutations

If STR typing is used for establishing family relationship among individuals as part of a forensic investigation, it is expected that there is an awareness of the probability of mutations in the evaluation of the results. Whenever a genetic inconsistency is observed, a probability of a mutation should be incorporated based on the recommendations of Gjertson et al. [53]. Examples of this type of identification include the comparison of the DNA of an off-spring to known parents to determine if it is captive bred or captured from the wild. The probabilities of mutations at the loci selected can be established by family studies. These parameters are of especial value in cases where a single or few genetic inconsistencies with the relationship in question are observed together with a relatively high likelihood ratio. These results can (a) be obtained in situations where other close genetic relationships exist, rather than the relationships in question or (b) be explained by mutations.

Recommendation #9: *In relationship testing, the mutation probabilities of the STR alleles should be estimated if encountered, or at least the probability of a mutational event occurring should be considered when there is genetic inconsistency at a single or few loci while all other loci show genetic consistency.*

9. Allele frequency databases

A reasonable number of individuals should be typed in order to estimate the allele frequencies. The individuals collected should be representative of the population from which the unknown sample may have originated. Sufficient samples need to be collected such that it is possible to account for any sampling error [54]. Often 200 members of a representative population are sampled as a *de facto* standard [54], although the size of samples is dependent on the number of potential contributors and the locus diversity levels. The frequency (reference) databases should be examined for Hardy–Weinberg equilibrium and any deviation noted.

Recommendation #10: *Relevant population and forensic genetic parameters including allele frequencies should be estimated.*

10. Kinship effects

A factor that affects the chance that two members of the same population share an allele as they have a common genetic ancestor is used commonly in forensic genetic comparisons of human DNA profiles. Such a kinship factor, often named F_{ST} or θ, typically ranging from 0.01 to 0.03 in humans, is often applied. The magnitude of the figure used indicates the estimated degree of common ancestry. In large human populations, the degree of common ancestry is typically small, but this may not be the case in non-human populations, particularly in isolated small wild animal populations, domesticated populations, species that do not disperse, or species that mate polygamously. Kinship factors for wild creatures, such as deer, bear and European badger have been published and indicate the amount of inbreeding within wild populations for these species. An accurate kinship factor should be calculated and applied for each population where possible. The importance and use of the *F*-statistics is standard in both wild animal and human populations [55] and has been reviewed recently [56].

Recommendation #11: *A kinship factor should be determined and applied in any calculation. The type of kinship factor applied should be stated clearly and justification should be made for the factor incorporated.*

11. Reporting of results

The format of any report will depend on the criminal justice system. Any report should state the purpose of the investigation

and on whose behalf it was written. It is important that any report states clearly the allegation being considered.

The scientific basis of the test should be provided in the report.

The results obtained should be disclosed and reported. The results should be based on information within a casefile that should be comprehensive, including all material collected and produced as part of the analysis. The casefile should be open to review by an expert if requested.

The method of the evaluation of the weight of the evidence should be stated clearly and an expert opinion provided given the allegation and any credible alternatives. Probabilistic statements based on likelihood ratio principles should be supported by studies published in international peer reviewed publications. If this is not possible, the relevant data should be open to experts if requested.

Recommendation #12: *A comprehensive casefile should be maintained. A likelihood ratio approach is the recommended way to evaluate the weight of the evidence, considering more than one proposition.*

12. Accreditation of laboratories working of non-human DNA typing for forensic purposes

Those laboratories that aim to undertake testing routinely should consider accreditation to the ISO17025 standard as recommended by the ISFG [57]. This recommendation is particularly aimed at those laboratories that conduct tests on non-human (animal) DNA for forensic purposes on a routine basis. If a laboratory is conducting such testing on an infrequent basis, the recommendations set out in this report should be adhered to where appropriate and adherence to the recommendations of the Commission stated.

Recommendation #13: *Accreditation should be sought if DNA testing of non-human animal DNA for a particular purpose is to be become routine.*

13. Concluding comments

Ideally, the recommendations concerning forensic genetic investigations of non-human DNA are identical to those of investigations of human DNA. The commission, however, is aware of the fact that the present knowledge of the genetics of non-human DNA is limited although such knowledge is increasing rapidly. The ISFG recommendations are designed to indicate the complex nature of such analyses and that there are similarities with the examination of human DNA. The recommendations are not designed to be restrictive such that laboratories opt not to take on non-human DNA testing, rather good practice is encouraged. The same approach should be undertaken when examining any item, be it for human identification or for analysis of non-human DNA.

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